Case No. 4380US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Russell G. Higuchi**	)	Group Art Unit: 1743
Serial No.: 08/968,208	)	Examiner: J. Snay
Filed: November 11, 1997	)	Confirmation Number:
For: INSTRUMENT FOR MONITORING NUCLEIC ACID AMPLIFICATION	)	

DECLARATION OF RUSSELL G. HIGUCHI, Ph.D. UNDER 37 CFR § 1.131

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, RUSSELL G. HIGUCHI, Ph.D., declare as follows:

1. I am the inventor of the above-captioned patent application and am familiar with its contents and currently pending claims.

2. I received a Doctor of Philosophy degree in Molecular Biology at the University of California, Los Angeles in 1980.

3. At the time I made the instrument claimed in the above-captioned patent application, I was a Scientist at Cetus Corporation.

4. Prior to April 15, 1991, the invention claimed in the above-captioned application was actually reduced to practice in an experiment carried out to confirm that my proposal to monitor PCR reactions in real time was possible, and the results of the experiment were faxed to me. I

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placed a copy of the experiment results that were faxed to me into my notebook on or before April 15, 1991.

5. In the experiment, amplification of Y-chromosome-specific repeat sequences by a polymerase chain reaction (PCR) carried out in the presence of ethidium bromide ("EtBr") was monitored in real time using an embodiment of an instrument claimed in the captioned application. The PCR reaction (100 µl in a 0.5 ml polypropylene centrifuge tube with its cap removed) contained: 10 mM Tris-HCl, pH 8.3; 4 mM $MgCl_2$; 2.5 units *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT); male DNA-specific primers (15 pmole each primer); 20 ng human male target DNA; and 0.5 µg/ml EtBr. The reaction was overlayed with mineral oil (2 drops) to prevent evaporation. Continuous monitoring of the PCR in progress was accomplished using a Fluorolog-2 fluorometer (SPEX, Edison, NJ) equipped with a fiber optic accessory (SPEX cat. no. 1950) to both send excitation light to, and receive emitted light from, the PCR tube. The probe end of the fiber optic cable was attached with "5-minute-epoxy" to the open top of the PCR tube, effectively sealing it. The exposed top of the PCR tube and the end of the fiber optic cable were shielded from room light and the room lights were kept dimmed during the PCR amplification. Thermocycling and fluorescence measurement were started simultaneously. Thermocycling proceeded for 30 cycles at 94 °C for 1 min. and 50 °C for 1 min. in a model 480 thermocycler (Perkin-Elmer Cetus) using a "step-cycle" program. Excitation was at the 500 nm band with about 2 nm bandwidth with a GG 435 nm cut-off filter (Melles Grist, Inc., Irvine, CA) to exclude second-order light. Emitted light was detected at 570 nm with a bandwidth of about 7 nm. An OG 530 nm cut-off filter was used to remove the excitation light. A time-base scan with a 10 second integration time was used and the emission signal was ratioed to the excitation signal to control for changes in light-source intensity. Data were collected using the dm3000f, version 2.5 (SPEX) data system.

6. A copy of the results of the experiment, which I received and understood prior to April 15, 1991, is attached hereto as EXHIBIT A. The fluorescence trace as a function of time clearly shows amplification of the target DNA. Fluorescence intensity is minimum at the denaturation temperature (94 °C) and maximum at the annealing/extension temperature (50 °C). The fluorescence maxima at the annealing/extension temperature begins to increase at about 4000 seconds of thermocycling, and continues to increase with time, indicating that increasing

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amounts of double-stranded DNA amplification product are being produced as a function of time. The fluorescence minima at the denaturation temperature do not significantly increase, presumably because at this temperature there is no double-stranded DNA for EtBr to bind.

7. On the fluorescence trace as faxed to me, it is written that the annealing/extension temperature is 55 °C. The correct annealing/extension temperature of the experiment, as indicated above, is 50 °C. When I placed a copy of the experiment results that were faxed to me into my notebook, I corrected the temperature that was written on the trace based on information I received after the experiment was conducted. In any event, the results of the experiment would be the same whether the annealing/extension temperature had been 50 or 55 degrees as both are appropriate temperatures for annealing/extension of the particular primers used.

8. On or before April 15, 1991 a control PCR experiment was carried out without DNA. The control experiment further confirmed to me that the experiment worked.

9. At some point after this experiment was performed, I prepared a manuscript that describes exactly the steps performed in the experiment and includes a fluorescence trace as Figure 5 of a subsequent run of the exact same experiment. I submitted this article to the scientific journal *BioTechnology* for publication. A copy of the subsequently published article is, Higuchi *et al.*, 1992, *BioTechnology* 10:413-417, is attached hereto as EXHIBIT B.

10. I declare further that all statements made herein of my own knowledge are true, that I believe that any statements herein made on information and belief are true, and that I acknowledge that any willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and may jeopardize the validity of the application or any patent issuing thereon.

Date: October 6, 2003

Russell G. Higuchi, Ph.D.

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